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Production of Milk-Clotting Protease from *Bacillus subtilis*

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Abstract An indigenous *Bacillus subtilis* strain isolated from soil was found to be a potent milk-clotting protease (mcp) producer. Production optimized using response surface methodology (RSM) yielded 1,190 U/ml of enzyme in medium containing 6% fructose, 1% casein, 0.3% NH_4NO_3, 10 mM $CaCl_2$, pH 6.0 and inoculated with 3% inoculum and incubated at 250 rpm for 72 h. Solid-state fermentation resulted in 1,080 and 952.3 U/gds of milk-clotting protease using soybean meal and rice bran, respectively, with higher proteolytic values of 18.97 and 9.1 IU/gds. Production in a biphasic system using an overlay of RSM-optimized medium on solid layer of 6% fructose and 1% casein with 1.5% agar resulted in significant enzyme production. Maximum mcp was obtained using a biphasic system where solid: liquid ratio of 3.0 resulted in a final yield of 1,276.65 U/ml with a yield index of 1.80 as compared to static liquid culture. However, significant increase or difference was noted as compared to yield obtained after RSM. This is the first report on the use of RSM for production of mcp from a bacterial species.

Keywords Milk clotting protease · Response surface methodology · Solid-state fermentation · Biphasic system

Abbreviation

MCA	milk-clotting activity
PA	proteolytic activity
milk-clotting protease	mcp
SmF	submerged fermentation
SSF	solid-state fermentation
RSM	response surface methodology
FCCCD	face-centered central composite design

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Introduction

Rennin, a milk-curdling enzyme used in cheese making, is obtained from the stomach of the unweaned calf. Shortage of animal rennet supply has led to tremendous efforts in seeking out alternatives to calf rennet [1]. Enzymes from microorganisms like *Mucor miehei*, *Mucor pusillus*, *Endothia parasitica*, *Irpex lacteus*, and *Aspergillus niger* have replaced calf chymosin in commercial cheese making [2]. In published studies, the effects of medium composition and concentration of each constituent on milk-clotting activity of some bacteria, and different fungi have been investigated mostly in batch, submerged batch, or solid-state systems [3]. Commercially, the optimization of medium is done to maintain a balance between the various components, thereby, minimizing the amount of unutilized components at the end of fermentation. Research efforts have been directed mainly towards evaluating the effect of various carbon and nitrogen sources, requirement of divalent metal ions and optimization of environmental and fermentation parameters such as pH, temperature, aeration, and agitation on milk-clotting protease (mcp) yield [4]. No defined medium has been established for the optimum production of mcp from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production.

In our preliminary studies for the development of a production medium by one at a time strategy, maximum mcp production of 571.43 U/ml was observed. Casein, fructose, inoculum size, and agitation rate were the factors which significantly affected production. In the present investigation, three different strategies were investigated for mcp production from *Bacillus subtilis*. The first strategy evaluated was response surface methodology wherein the interactive effects of the most significant factors affecting enzyme production in submerged conditions was carried out followed by investigations on production in solid-state fermentation. Lastly, biphasic system was evaluated for production. A comparative evaluation of the three strategies has been presented in the present investigation.

Materials and Methods

Organism and Culture Conditions

The organism, *Bacillus subtilis* obtained after extensive screening [5] was maintained on nutrient agar slants (pH 7.0) at 10±1 °C in a bacteriological incubator with periodic subculturing.

Four percent inoculum grown in nutrient broth (pH 7.0) at 37±1 °C was used for inoculating the production medium containing (% *w*/*v*) fructose 4; casein 0.75; ammonium nitrate 0.3; K_2HPO_4 0.1; KH_2PO_4 0.05; and $CaCl_2$ 10 mM; pH was adjusted to 6.0 using 1 M NaOH/HCl before sterilization (10 psi and 20 min). Samples were harvested at 72 h, centrifuged at 8,000×*g* for 15 min, and the supernatant obtained was assayed for milk clotting activity (MCA) and proteolytic activity (PA).

Production Under Submerged Conditions (Response Surface Methodology)

Experimental Design for Milk Clotting Protease Production

The conventional one at a time method was used to select the effective factors and the initial test range of each of the selected variables: fructose and casein concentrations,

inoculum size and agitation rate. Taking these factors into consideration, a response surface methodology using face centered central composite design (FCCCD) was adopted for improving mcp production from *B. subtilis*. The statistical software package (Design Expert® 6.0, Stat-Ease, Inc., Minneapolis, USA) was used to analyse the experimental design. Each factor was studied at three different levels (-1, 0, +1). All factors were taken at a central coded value considered as zero (Table 1) and a matrix of 30 experiments was generated using the software package (Table 2). The average MCA (U/ml) was taken as dependent variable or response (Y). A second-order polynomial equation was then fitted to the data by multiple regression procedure. This resulted in the independent factors. All tests were performed in triplicates, and the data represented is a mean of the three. For a four-factor system, the model equation was:

$$Y = \beta 0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD \quad (1)$$

where Y is the predicted response; β_0, intercept; β_1, β_2, β_3, β_4, linear coefficients; β_{11}, β_{22}, β_{33}, β_{44}, squared coefficients; β_{11}, β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, β_{34}, interaction coefficients.

Validation of the Model

The model was validated by considering different permutation and combination of medium components, selected within the model range so as to fit the second-order polynomial equation. Six sets of experiments were generated and carried out.

Solid-State Fermentation

Ten grams wheat bran was taken in a series of 250-ml Erlenmeyer flask and moistened with 30 ml of phosphate buffer (0.01 M, pH 6.0) containing 0.25% *w/v* casein to obtain a moisture ratio of 1:3.0. The flasks were sterilized at 121 °C for 60 min. Flasks were inoculated with 3% inoculum (OD_{660}=0.6–0.8) and incubated at 37 °C for 96 h.

Table 1 Experimental range and levels of the independent variables used in RSM in terms of actual and coded factors.

Range of levels						
Variables	Actual	Coded	Actual	Coded	Actual	Coded
Fructose[a]	2.0	–1	4.0	0	6.0	+1
Casein[a]	0.5	–1	0.75	0	1.0	+1
Inoculum size[b]	3.0	–1	4.0	0	5.0	+1
Agitation size (rpm)	150	–1	200	0	250	+1

[a] Values are in % *w/v*

[b] Value is in % *v/v*

Table 2 Experimental design (coded) of experiments with experimental and predicted response.

Run order	Fructose	Casein	Inoculum density	Agitation rate	Mean observed response (U/ml)	Predicted response (U/ml)
1	–1	–1	–1	+1	546.0	548.93
2	+1	+1	+1	–1	1030.0	1028.04
3	0	0	0	0	568.0	576.06
4	–1	+1	+1	–1	653	654.24
5	+1	–1	+1	–1	768.0	763.4
6	0	0	0	0	578.0	576.06
7	+1	+1	+1	+1	1180.0	1191.62
8	+1	–1	–1	+1	923.0	920.24
9	–1	–1	+1	–1	386.0	398.60
10	+1	+1	–1	–1	1040.0	1035.29
11	–1	+1	–1	+1	813.0	816.07
12	–1	–1	–1	–1	435.0	421.85
13	–1	+1	–1	+1	815.0	806.82
14	–1	+1	–1	–1	674.0	674.99
15	0	0	0	0	574.0	576.06
16	+1	–1	–1	–1	773.0	782.15
17	+1	+1	–1	+1	1190.0	1187.37
18	0	0	0	0	582.0	576.06
19	+1	–1	+1	+1	913.0	912.99
20	–1	–1	+1	+1	525.0	528.18
21	0	0	+1	0	567.0	562.12
22	0	–1	0	0	513.0	514.67
23	0	0	0	–1	525.0	534.45
24	0	0	0	+1	687.0	679.78
25	0	0	–1	0	569.0	576.12
26	–1	0	0	0	497.0	503.34
27	+1	0	0	0	880.0	875.89
28	0	0	0	0	584.0	574.54
29	0	+1	0	0	780.0	780.56
30	0	0	0	0	574.0	574.54

Enzyme Extraction

The enzyme from the fermented bacterial bran was extracted twice with phosphate buffer (0.01 M and pH 6.0) and filtered. The total pooled filtrate (50 ml) of two extractions was centrifuged (8,000×*g*, 20 min, 4 °C) and used as the source of enzyme.

Optimization of Production

Different solid supports like wheat bran, rice bran, coarse soybean meal and crushed, corn cob were evaluated for mcp production. The effects of the following moistening agents were evaluated on production: (a) 30 ml of phosphate buffer (0.005 M and pH 6.0), (b) distilled water, and (c) salt solution (composition g/l): ammonium nitrate 0.3; K_2HPO_4 0.1; KH_2PO_4 0.05; $CaCl_2$ 10 mM; and pH 6.0. Different concentrations of casein and skim milk powder were evaluated for the effect on milk-clotting protease production.

Production in a Biphasic System

Production under static biphasic system was evaluated in three sets in 250-ml Erlenmeyer flasks with a final volume of 100 ml in each flask.

The composition of the sets are as follows:

Medium	Solid phase	Liquid phase
Set I	RSM optimized medium +1.5% agar	RSM optimized medium
Set II	Fructose (6%), casein (1%), and agar agar (1.5%)	RSM optimized medium
Set III	RSM optimized medium +1.5% agar	Fructose (6%), casein (1%)0

The ratio of solid to liquid phase was optimized. The bacterial growth and production in the biphasic medium was numerically compared to the results in the control flasks having equal volume of liquid production medium.

Analytical Methods

Assay for Milk-Clotting Activity

Milk-clotting activity was carried out by the method of [6]. The amount of enzyme required to clot milk in 1 min is defined as containing 400 MCA units [7].

MCA (U/ml)$=400\times t^{-1}\times$DF; where t is time in minutes required for clotting and DF is the dilution factor.

Assay for Proteolytic Activity

Proteolytic activity was estimated by modified Anson's method as described by Arima et al. [6]. One unit of proteolytic activity is defined as the amount of enzyme which yields the color equivalent to 1 μmole of tyrosine per minute.

All experiments were run in triplicate, sets repeated with similar results, and mean values presented. The values, when analyzed by Student's t test, were within the 95% confidence limit.

Results and Discussion

Previous investigations revealed that this *Bacillus subtilis* strain could produce mcp and was amenable to process optimization. A final yield of 571.43 U/ml was obtained after one at a time optimization under submerged conditions [5]. Therefore, in the present investigation, three strategies for production viz. RSM (in submerged conditions), SSF, and biphasic systems were evaluated.

Production Under Submerged Fermentation Using Response Surface Methodology

Four factors, i.e., fructose and casein concentrations, inoculum size, and agitation rate were found to be the most significant affecting production of mcp under submerged conditions,

but their possible interactions were not evaluated. Thus, a statistical design, response surface methodology was employed here to study their possible interactions for effect on enzyme production. The results of the RSM experiments, both the predicted and experimental data, are presented in Table 2. Consequently, the data was fitted with a second-order polynomial function to get the following regression equation which is an empirical relationship between the logarithmic values of enzyme yields and test variables in coded unit: (Eq. 2).

$$Y = 575.30 + 186.28 \times A + 132.94 \times B - 7.00 \times C + 72.67 \times D + 115.08 \times A^2 + 73.08 \times B^2 - 5.42 \times C^2 + 32.58 \times D^2 + 0.00 \times A \times B + 3.38 \times A \times C + 2.75 \times A \times D + 2.88 \times B \times C + 3.50 \times B \times D + 2.88 \times C \times D. \quad (2)$$

Where Y: MCA (U/ml), A: fructose; B: casein; C: inoculum size; D: agitation rate.

Analysis of variance (ANOVA) was employed for evaluating the data obtained (Table 3). It indicated that the model terms of A, B, C, D, A^2, B^2, and D^2 were significant ("probe > F" less than 0.0500) for production of mcp.

The regression equation obtained from the ANOVA showed that the multiple correlation coefficient (R^2) was 0.9991 (a value >0.75 indicates fitness of the model) [8]. This is an estimate of the fraction of overall variation in the data accounted by the model. This model is capable of explaining 99.91% of the variation in response. The "adjusted R^2" is 0.9983 and predicted R^2 is 0.9947, indicating that the model is good as for a good statistical model, the R^2 should be in the range of 0–1.0, and the nearer to 1.0 the value is, the more fit the model is deemed to be [9]. The "adequate precision value" of the present model was 127.536, suggesting that the model can be used to navigate the design space. The "precision value" is an index of the signal-to-noise ratio, and values of higher than 4 are prerequisites for a model to be a good fit [10]. The model showed standard deviation, mean, and predicted residual sum of squares values of 8.61, 704.73, and 6.42038E+005. The lower value of coefficient of variation (C.V.=1.22) showed that the experiments conducted were precise and reliable [9, 11].

The parity plot showed a satisfactory correlation between the experimental and predictive values (Fig. 1), wherein, the points clustered around the diagonal line indicates a good fit of the model [12]. In order to determine the optimal levels of each factor for maximum mcp production, three dimensional response surface plots were constructed. Figure 2 shows the response for the interactive factors; fructose and casein when inoculum density was at 3.0% and 250 rpm agitation rate. Maximum enzyme activity in this condition was predicted to be 1,186 U/ml. The production of mcp varied considerably over the range tested from 120.3–1,190 U/ml. Fructose and casein concentrations were found to be most influential for production. Any change in their concentrations resulted in a drastic change; yet, these two are clearly synergistic in action. An increase in mcp yield with increase in

Table 3 ANOVA for response surface quadratic model.

Model terms	Value
R^2	0.9991
Adj R^2	0.9983
Pred R^2	0.9947
Adeq precision	127.536
Model F value	1,164.20
Lack-of-fit value	2.24

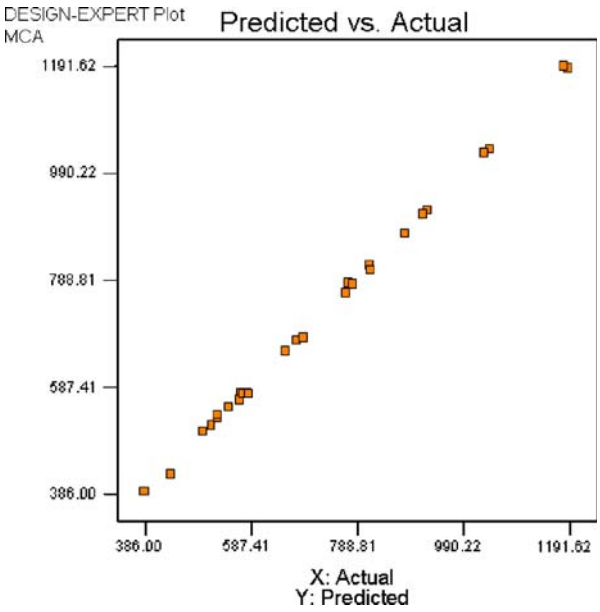

Fig. 1 Comparative plot showing predicted vs. observed values of milk-clotting protease from *Bacillus subtilis*

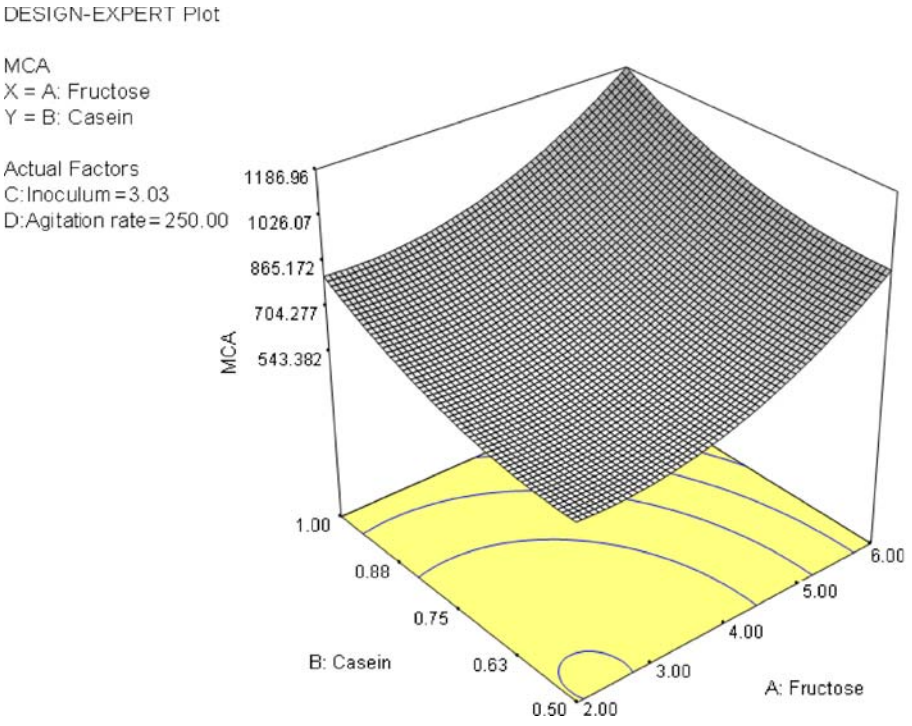

Fig. 2 Response surface curves of milk-clotting protease production from *Bacillus subtilis* showing interaction between casein and fructose

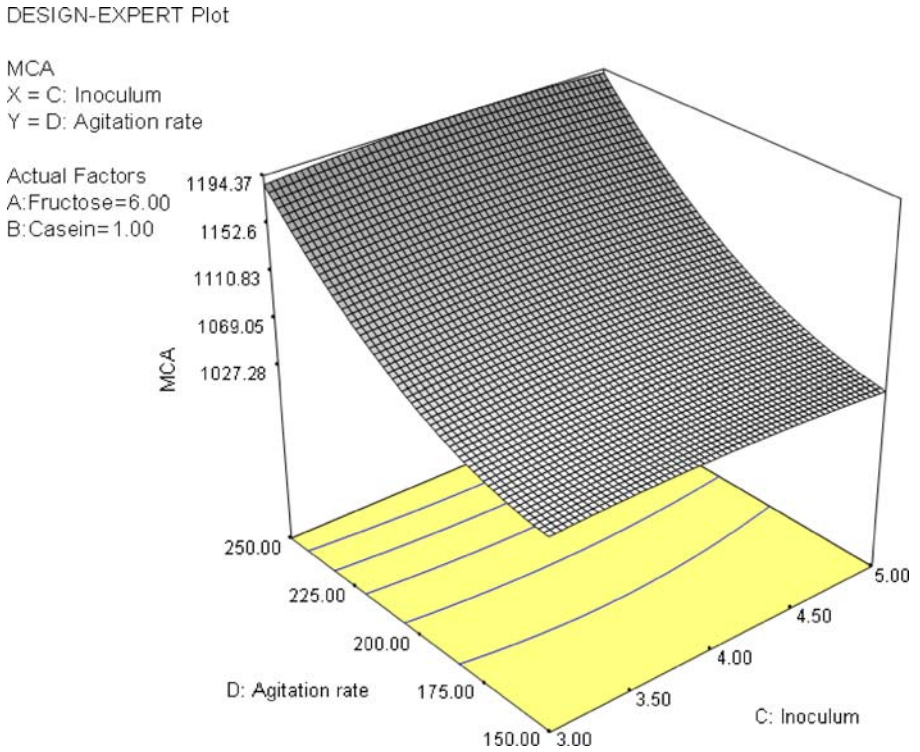

Fig. 3 Response surface curves of milk-clotting protease production from *Bacillus subtilis* showing interaction between inoculum density and agitation rate

fructose concentration versus casein concentration was observed. Previously, it was observed that casein is necessary for the induction of this enzyme but presence of fructose doubled the yield [5]. Figure 3 indicates the negative effect of inoculum density on milk clotting protease production while the response varied as a function of agitation rate (Fig. 4). On the contrary, Ayhan et al. [13] have reported using 5.2% (*v*/*v*) of inoculum for

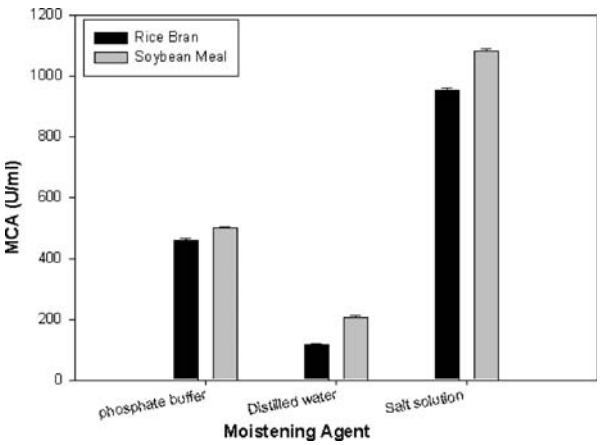

Fig. 4 Effect of different moistening agents on mcp production in SSF

production of rennin in a chemically defined medium after using a similar statistical model for optimization.

Validation of the Model

Validation of the model showed that experimental values were found to be very close to the predicted values (Table 4). Validation of the statistical model and regression equation was performed by taking fructose (6.0%), casein (1.0%), agitation rate (225.0 rpm), and inoculum level (3.0%) in the experiment. The predicted response of 1,116.97 U/ml was close to the actual experimental response of 1,118.0 U/ml, thus, proving validity.

Production Under Solid-State Fermentation

In solid-state fermentation, milk clotting protease production has been investigated mostly using wheat bran [7, 14–17]. Therefore, wheat bran as solid support for mcp production was evaluated. Production carried out with moistening agent supplemented with 0.25% casein yielded 215.0 U/g of MCA. There was no production of mcp in absence of casein (data not shown) [5].

On investigating different solid supports for mcp production by this *B. subtilis*, it was observed that, rice bran and soybean meal supported maximum mcp yield using phosphate buffer as moistening agent supplemented with 0.25% casein. Though, rice bran has been reported by Thakur et al. [14] for mcp production from *Mucor*, but maximum yield was obtained only with wheat bran. Soybean meal has been reported to be a supporter of MCA by Cavalcanti et al. [1] but in submerged fermentation only. Replacing phosphate buffer with a salt solution increased mcp production in both rice bran and soybean meal by 2.06- and 2.17-folds, respectively (Fig. 4). However, PA increased by 3.84- and 5.97-folds, respectively, for rice bran and soybean meal. Maximum mcp production was observed in presence of 0.25% casein and 1% skim milk powder for both rice bran and soybean meal systems. Casein is a component of skim milk, and skim milk has been reported as an inducer of mcp production [14, 18, 19]. However, casein was a better inducer as compared to skim milk powder to mcp production (Table 5).

The major drawback observed in this system was the noticeably higher PA leading to a lower MCA/PA ratio. The PA estimated for the mcp produced under the optimal conditions after RSM (Table 2) was approx. 1.00 IU/ml (data not shown) which resulted in a MCA/PA ratio of 1190. This ratio is far more than that obtained for mcp production in SSF.

Table 4 Validation model.

Fructose (%)	Casein (%)	Agitation rate (rpm)	Inoculum level (%)	Predicted Value	Experimental Value
3.0	0.63	250	3.0	563.68	567.0
5.0	0.88	250	3.0	859.40	865.0
5.5	0.75	225	3.0	788.90	793.0
6.0	0.88	175	3.0	922.69	917.0
6.0	1.0	225	3.0	1,116.97	1,118.0
5.0	1.0	175	3.0	877.10	869.0

Table 5 Effect of inducers on milk clotting protease production.

Inducers	Rice bran			Soybean meal		
	MCA (U/ml)	PA (IU/ml)	MCA/PA	MCA (U/ml)	PA (IU/ml)	MCA/PA
Casein						
0.25 (control)	952.3	9.1	104.99	1080.0	18.97	56.93
0.50	950.7	9.72	97.81	1060.0	19.74	53.69
0.75	900.0	11.98	75.13	975.0	17.80	54.78
1.00	835.6	13.07	63.93	915.7	15.63	58.59
1.25	790.4	10.41	75.93	852.3	13.07	65.21
Skim milk powder						
0.25	434.4	5.40	80.44	487.3	5.50	88.60
0.50	571.0	6.42	88.94	615.7	8.82	69.81
0.75	698.3	7.34	95.14	780.0	14.97	52.10
1.00	828.3	8.79	94.23	934.6	20.00	46.73
1.25	734.7	8.90	82.55	845.4	18.07	46.79

Production Under Biphasic System

Biphasic system was initially developed to concentrate bacterial cells by growing them in a confined environment with diffusional access to a reservoir of nutrients. Hestrin et al. [20] used the system for the production of bacterial levansucrase while Kaur et al. [21] have reported using this system for protease production from *Bacillus* sp. P-2. Maximum mcp of 934.6 U/ml was produced in 72 h with 846.0 and 667.1 U/ml in Sets I and III, respectively (data not shown). Tyrell et al. [22] reported that the ratio between the volume of agar and broth and the depth rather than the quantity of agar were critical in a biphasic system. They reported a favorable yield index at an agar broth ratio of 4. Similarly, in the present investigation on increasing this ratio to 3 from 1, maximum mcp yield of 1,276.65 U/ml was obtained (Table 6) which is 1.36-fold higher than initial yield (934.6 U/ml) and 1.073-fold higher than yields obtained after RSM. Kaur et al. [21] have, however, reported much higher fold production which is probably due to a slow release of nutrients from the lower solid phase of the medium, which support the growth of the organism when the medium is exhausted just as in batch culture. Similarly Gupta et al. [23] have also reported significant xylanase production using *Staphylococcus* sp. SG-13. On the contrary, Tyrell et al. [22] have reported that in spite of addition of nutrients to the agar or supplementation of oxygen failed to increase production further. This may be due to the possibility that some growth

Table 6 Production of milk clotting protease by *Bacillus subtilis* in biphasic growth medium at pH 6.0 and 37 °C under static conditions after 72 h.

Solid/liquid ratio	CFU/ml ($\times 10^{10}$)	MCA (U/ml)	Yield Index
0	1.3	257.56	1.0
1	1.8	934.6	1.32
2	2.7	1,048.3	1.48
3	4.0	1,276.65	1.80
4	4.2	1,200.0	1.69
5	4.5	1,069.3	1.51

requirement, possibly oxygen diffusion, had become limiting and that there is no arbitrary maximum cell density that will limit growth, a notion sometimes inferred from Bail's concept of an "M concentration" [22].

The results of production in the biphasic system were numerically compared to the results in the control flask having the same volume of broth as both biphasic layers. The degree of concentration index; that is, how many times more concentrated the enzyme was in the biphasic culture than in the equivolume broth control was also estimated. The comparison of total yields was represented as a yield index; that is, the ratio of the total yield in the biphasic flask to the total yield in the equivolume broth control. There was 4.96-fold increase in mcp values as compared to the production in static broth conditions. A 12-fold increase in xylanase production has been reported by Gupta et al. [23] in a biphasic system as compared to the static liquid culture.

Conclusions

Production of milk clotting protease was investigated using three strategies. 1,190 U/ml of mcp was obtained after optimization of production by RSM in submerged culture which is 2.08-fold higher as compared to one at a time yield of 571.43 U/ml. SSF yielded 1,080 and 952.3 U/gds mcp on soybean meal and rice bran as solid substrates, respectively, with 0.25% casein acting as inducer. However, this system yielded considerable higher proteolytic value of 18.97 and 9.1 IU/gds on soybean meal and rice bran substrates, respectively, which reduced the MCA/PA values considerably. The biphasic system on the other hand yielded maximum mcp titers of 1,276.65 U/ml with a yield index of 1.80 as compared to static liquid culture. This is also 2.23-fold higher as compared to one at a time yield and 1.07-fold higher than RSM yield. Therefore, RSM and biphasic strategies lead to significant increase in mcp yields as compared to initial yield of 571.43 U/ml. SSF on the other hand, though gave comparable yield, was lesser than the other two. Finally, this is one of the few studies carried out for studying the influence of production parameters on increasing milk-clotting production through statistical methods and in SSF or biphasic systems. To the best of the authors' knowledge, the only reference to the use of statistical design for mcp production has been reported by Ayhan et al. [13] for *Mucor* sp.

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